Bacteria", Pediatric Pulmonology , Supplement 5 (Abstracts), October 1990, Abstract No. 8, page 189. Please note that this reference is **not prior art** to the present application since it was published well after the March 5, 1990 filing date of Applicants' 07/488,307 application whose priority is claimed. This abstract shows concisely the value of Applicants' discoveries, and that the scientific barriers, which Applicants' surmounted by three different approaches as set forth in this application, were still being experienced by others **after** Applicants' priority filing date. Interestingly, these "others" share commonality with the authors and/or inventors of the key references of record upon which previous art rejections have been made.

Specifically, the abstract shows unambiguously that great difficulties prevented assembly of a construct capable of encoding a complete CFTR and stable as a recoverable clone. **No solutions** to these difficulties were provided. In particular, the Examiner's attention is respectfully directed to the following statements made by the authors regarding their efforts: (1) "conventional approaches to cloning are unlikely to work", (2) "numerous other strategies have also been attempted without success", and (3) "reducing the quantity of product by switching from a high copy number vector (Bluescript) to a lower copy number vector (about 20 copies per cell) of the pBR322 type yields rearranged clones also".

With respect to item (3), the reference specifically **teaches away** from the utility of low copy number methodology which, by that time, had **already been disclosed in the present Applicants' patent application,** along with two other methods, as effective to stabilize the CFTR DNA for cloning in bacterial cells. Indeed, Applicants discovered, as set forth on pages 12-13 of the specification that truly lower copy number was effective.

Additionally, to address the notion that low copy number technology may be obvious (raised in Examiner's paper of January 21, 1993 at page 4, lines 7-28 in the 07/488,307 parent hereof), Applicants respectfully observe that such an argument improperly uses the Applicants' own discoveries as references against themselves. This approach has been uniformly and consistently deemed improper by the CAFC.

Specifically, the referenced rejection relied upon Harris, T.R.J., "Expression of Eucaryotic Genes in E. coli", in Genetic Engineering 4, R. Williamson ed., 1983, Academic Press, New York, pp. 127-185. However, this reference is concerned primarily with problems that interfere with intended polypeptide expression in E. coli . This should be seen in contrast to the present situation where host bacterial cells were intended merely as a vehicle to propagate/maintain an encoding DNA, but without expression therefrom. Indeed, the 1983 Harris reference, obviously, was of no assistance to the authors of Drumm et al. since those authors, certainly in possession of the current art including Harris, nonetheless recovered only "primarily grossly rearranged constructs". Thus Applicants respectfully submit that the obviousness rejection may be properly withdrawn.

Finally, with respect to the Examiner's concern (see the Office Action of June 16, 1994 at page 3 thereof) that the term "cellular recombination" as used in Applicants' Specification is indefinite, consideration of the following comment is respectfully requested. The term cellular recombination was intended to refer, for example, to those intracellular processes that prevented, or contributed to, the art-recognized failure to recover stable bacterial clones of non-rearranged CFTR-encoding DNA. In reviewing the specification on this issue, Applicants discovered the unintended deletion of relevant language and with this amendment ask for the Examiner's indulgence in reinserting it into the specification. It was present in the original priority application (page 9, lines 16-28 of the 07/488,307 application). Applicants would also suggest such incorporation is proper since Applicants requested, when the present application was filed, that the text of the earlier applications be incorporated by reference.

Conclusion

Applicants firmly believe that the application is fully in condition for allowance, and that all pertinent issues have been addressed. It is respectfully requested that the claims now be passed to allowance. The Examiner is invited to phone either the undersigned at (508) 872-8400, or Applicants' counsel of

Supplement 5

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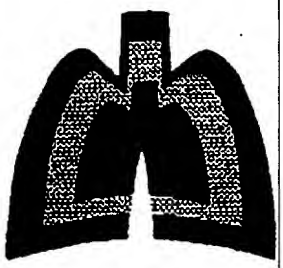

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supernatant. Solubilized extracts were added to a Reactive Brown-agarose column, washed and eluted with 5mM ATP to displace ATP-binding proteins. In 3 experiments, of the total activity recovered, 13.8±3.0% was in a single fraction containing 4.6±1.5% of the protein applied representing an increase in specific activity of 4.5-fold. A more general method for detecting ATP-binding proteins has also been developed in which rER membranes (20-30ug/ml) were photolysed with a mercury-arc lamp ±75uM 8-azido-ATP. Compared to unphotolysed membranes, loss of Ca^{2+},Mg-ATPase activity was 28.7% (n=4) in the absence of 8-azido-ATP but 72.7% (n=2) in its presence. The data suggest that detection of ATP-binding proteins by photoaffinity labelling coupled with Dye-agarose chromatography and ATPase measurements will be important tools in detection and purification of CFTR-like proteins in subcellular fractions from epithelial cells. This work was supported by the MRC.

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THE FULL-LENGTH CFTR cDNA IS TOXIC IN BACTERIAL HOSTS. M.Drumm, K. Murphy, H. Pope and F.Collins, Departments of Internal Medicine and Human Genetics and the Howard Hughes Medical Institute, Univ. of Michigan, Ann Arbor, MI, USA

In order to carry out in vitro and in vivo expression studies on CFTR, it is desirable to have a cDNA clone of the gene which is full length, identical in sequence to the mRNA. Typically, such clones are constructed and propagated in an E. coli host. The full-length clone for CFTR appears to be toxic to E. coli cells, however, so that conventional approaches to cloning are unlikely to work. Using the fragments of the cDNAs10-1, T16-1 and T16-4.5 (Science 245:1066), we have attempted to construct full-length clones, but each trial has yielded primarily grossly rearranged constructs. Only two appeared to be full length and, after sequencing, were also found to have anomalies, one having a 57 bp deletion in exon 6b, the other a single nucleotide insertion in exon 8. To prove that these clones were not merely cloning artifacts, the deleted exon 6b was recloned in parallel with the normal sequence from T16-1. The deletion cloned with an efficiency over 80 fold higher than the normal sequence, and the resultant "normal" clones were also rearranged, whereas the deleted clones were stable. The type and spacing of these clone-stabilizing mutations suggest that at least a portion of CFTR is expressed in the bacterial host and that the unaltered product is toxic to the bacterium. Reducing the quantity of product by switching from a high copy number vector (Bluescript) to a lower copy number vector (~20 copies per cell) of the pBR322 type yields rearranged clones also. Addition of transcriptional terminators at the 5' end to shut off read-through or cryptic transcription from the vector is also ineffective. Likewise, antisense transcription from a lac promoter at the 3' end fails to block the toxic effect of the gene. Numerous other strategies have also been attempted without success. As a partial solution, however, we have been able to create small quantities of the full length clone by ligation of the deletion and insertion clones to each other at a unique AflII restriction site. This method generates sufficient amounts of template for in vitro transcription, the RNA of which is being injected into Xenopus laevis oocytes. Similarly, this approach allows the generation of sufficient cDNA quantities for small-scale transient, complementation assays in which the DNA is administered to the cell by microinjection.

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ANTIBODIES TO CFTR USED AS PROBES OF T-84 CELL MEMBRANE PROTEINS. C.M. Fuller, S. Ran, E.B. Gargus, and D.J. Benos. Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, AL, USA.

We have succeeded in raising rat polyclonal antibodies to synthetic peptides corresponding to regions of the cystic fibrosis transmembrane conductance regulator (CFTR) molecule. Peptides were synthesized by solid-phase methods, purified to >95% purity by reversed phase HPLC, and subsequently sequenced. Monospecific antibodies have been purified from antisera by using the peptides as affinity ligands attached to immobilized diaminodipropylamine via carbodiimide couplings at the C-terminal. These antibodies have been used as probes for membrane proteins of T-84 cells which appear to express the mRNA for CFTR at a high level. One antibody, α-105, corresponding to the first extracellular loop of the CFTR molecule between hydrophobic domains 1 and 2 (residues 105-115: IASYDPDNKEE), specifically identifies a protein band at an apparent molecular mass of 195 kDa on immunoblots of T84 plasma membrane proteins. Several other lower molecular mass bands appear on the immunoblots, but these bands are non-specific in that they are also recognized by non-immune rat IgG. A second antibody, α-785, raised against a sequence in the R-domain of CFTR (residues 785-796: RKTTASTRKVSL) specifically recognizes a prominent band at 70 kDa. In some of the immunoblots produced with this antibody, a second band at 50 kDa was also observed. Binding studies, using ^{125}I-labeled α-105, have shown an approximate two-fold increase in binding of the antibody to T-84 plasma membranes over iodinated rat IgG (4 experiments). Binding of ^{125}I-labeled α-105 to T-84 plasma membranes can also be inhibited by addition of peptide 105-115. These antibodies should prove to be useful ligands for the subcellular localization and the ultimate purification of the CFTR protein from T-84 cells. Supported by N.I.H. Grant DK42017 and by funds provided by the Cystic Fibrosis Foundation.

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Expression of the MDR P-glycoprotein in *E.coli*.

DR Gill, TA Keevil, SC Hyde, CF Higgins. ICRF Laboratories, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, U.K.

The CFTR protein, defects in which are responsible for CF, is related to the mammalian MDR protein. Multidrug resistance (MDR) is an important reason for the failure of chemotherapy in the treatment of advanced cancers. The MDR protein pumps chemotherapeutic drugs out of tumour cells resulting in multidrug resistance. CFTR and MDR are the only mammalian members of a highly-conserved superfamily of ATP-dependent transport proteins. In order to investigate the mechanism by which MDR functions in the cell, we are expressing the protein in *E.coli* using a plasmid expression vector. Site-specific mutagenesis was used to place the human *mdr-1* gene under the control of an *E.coli* promoter, allowing controllable expression of the MDR protein. The plasmid is stable in *E.coli* and the intact MDR protein can be detected in immunoblotting experiments. We are now optimising expression of MDR with a view to purifying the protein and raising antibody, particularly to the external domains. We are also attempting to induce the MDR protein to insert correctly into the *E.coli* cytoplasmic membrane and consequently provide a functional protein. Functional MDR in *E.coli* will allow us to apply genetic techniques to identify the protein domains responsible for substrate binding, to determine the normal substrate of this transporter, provide an assay for specific MDR inhibitors, and eventually, to elucidate the transport mechanism itself. We hope that this approach will contribute towards determining the molecular basis of the MDR phenotype and an understanding of the mechanisms by which the family of transporters work.

Enlarged

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THE FULL-LENGTH CFTR cDNA IS TOXIC IN BACTERIAL HOSTS. M.Drumm, K. Murphy, H. Pope and F.Collins, Departments of Internal Medicine and Human Genetics and the Howard Hughes Medical Institute, Univ. of Michigan, Ann Arbor, MI, USA

In order to carry out in vitro and in vivo expression studies on CFTR, it is desirable to have a cDNA clone of the gene which is full length, identical in sequence to the mRNA. Typically, such clones are constructed and propagated in an E. coli host. The full-length clone for CFTR appears to be toxic to E. coli cells, however, so that conventional approaches to cloning are unlikely to work. Using the fragments of the cDNAs10-1, T16-1 and T16-4.5 (Science 245:1066), we have attempted to construct full-length clones, but each trial has yielded primarily grossly rearranged constructs. Only two appeared to be full length and, after sequencing, were also found to have anomalies, one having a 57 bp deletion in exon 6b, the other a single nucleotide insertion in exon 8. To prove that these clones were not merely cloning artifacts, the deleted exon 6b was recloned in parallel with the normal sequence from T16-1. The deletion cloned with an efficiency over 80 fold higher than the normal sequence, and the resultant "normal" clones were also rearranged, whereas the deleted clones were stable. The type and spacing of these clone-stabilizing mutations suggest that at least a portion of CFTR is expressed in the bacterial host and that the unaltered product is toxic to the bacterium. Reducing the quantity of product by switching from a high copy number vector (Bluescript) to a lower copy number vector (~20 copies per cell) of the pBR322 type yields rearranged clones also. Addition of transcriptional terminators at the 5' end to shut off read-through or cryptic transcription from the vector is also ineffective. Likewise, antisense transcription from a lac promoter at the 3' end fails to block the toxic effect of the gene. Numerous other strategies have also been attempted without success. As a partial solution, however, we have been able to create small quantities of the full length clone by ligation of the deletion and insertion clones to each other at a unique AflII restriction site. This method generates sufficient amounts of template for in vitro transcription, the RNA of which is being injected into Xenopus laevis oocytes. Similarly, this approach allows the generation of sufficient cDNA quantities for small-scale transient, complementation assays in which the DNA is administered to the cell by microinjection.